EXHIBIT 3

l-Carnitine l-tartrate supplementation favorably affects markers of recovery from exercise stress

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L-Carnitine L-tartrate supplementation favorably affects markers of recovery from exercise stress

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Volek, Jeff S., William J. Kraemer, Martyn R. Rubin, Ana L. Gómez, Nicholas A. Ratamess, and Paula Gaynor. L-Carnitine L-tartrate supplementation favorably affects markers of recovery from exercise stress. *Am J Physiol Endocrinol Metab* 282: E474–E482, 2002; 10.1152/ajpendo.00277.2001.—We examined the influence of L-carnitine L-tartrate (LCLT) on markers of purine catabolism, free radical formation, and muscle tissue disruption after squat exercise. With the use of a balanced, crossover design (1 wk washout), 10 resistance-trained men consumed a placebo or LCLT supplement (2 g L-carnitine/day) for 3 wk before obtaining blood samples on six consecutive days (D1 to D6). Blood was also sampled before and after a squat protocol (5 sets, 15–20 repetitions) on D2. Muscle tissue disruption at the midthigh was assessed using magnetic resonance imaging (MRI) before exercise and on D3 and D6. Exercise-induced increases in plasma markers of purine catabolism (hypoxanthine, xanthine oxidase, and serum uric acid) and circulating cytosolic proteins (myoglobin, fatty acid-binding protein, and creatine kinase) were significantly ($P \leq 0.05$) attenuated by LCLT. Exercise-induced increases in plasma malondialdehyde returned to resting values sooner during LCLT compared with placebo. The amount of muscle disruption from MRI scans during LCLT was 41–45% of the placebo area. These data indicate that LCLT supplementation is effective in assisting recovery from high-repetition squat exercise.

hypoxia; ergogenic aid; resistance exercise; muscle damage

GIVEN THE OBLIGATORY ROLE of the carnitine system in transporting long-chain fatty acids to the mitochondrial matrix for oxidation (16), it is not surprising that there has been a great deal of research examining the potential of carnitine supplementation to enhance lipid oxidation, spare muscle glycogen, and improve exercise performance (3, 10). Despite the theoretical basis for usage of carnitine, the scientific literature has provided few "metabolic" benefits of carnitine supplementation for skeletal muscle during exercise, perhaps because of the difficulty of increasing carnitine concentrations in muscle with oral supplementation (2, 25). Using a different approach to study the effects of carnitine supplementation, Giamberardino et al. (7) demon-strated that eccentric exercise-induced delayed-onset muscle soreness and accumulation of creatine kinase during recovery was attenuated in subjects that supplemented with L-carnitine (3 g/day for 3 wk). Their findings indicate that carnitine supplementation may have a favorable effect on recovery from exercise. The study by Giamberardino et al., however, provided no data regarding potential mechanisms to explain the beneficial effect of carnitine supplementation.

This study was designed to further examine the role of carnitine supplementation in acute exercise stress and its influence on biochemical events during recovery. Carnitine has been shown to stimulate fatty acid oxidation in vascular endothelial cells (14). Ischemia in endothelial cells results in release of carnitine, increased oxidative stress, and compromised blood flow regulation, which can be overcome by intravascular carnitine administration (5, 15). Our working hypothesis was that carnitine supplementation could protect against carnitine deficiency in vascular endothelial cells and thereby improve blood flow regulation and delivery of oxygen to muscle tissue during and after exercise (18). The enhanced oxygen delivery was hypothesized to reduce the magnitude of exercise-induced hypoxia and thus attenuate the cascade of events that lead to purine catabolism, free radical formation, membrane disruption, and muscle soreness.

The biochemical events that occur with intense exercise are multiple and complex and involve catabolism of purines, generation of reactive oxygen species, and disruption of cell membranes. Performance of exercise with a strong eccentric component results in transient episodes of hypoxia, breakdown of ATP, accumulation of ADP within the smooth muscle of the precapillary sphincter, and activation of the enzyme adenylate kinase (24). Adenylate kinase catalyzes the formation of ATP and AMP from two molecules of ADP. Accumulation of AMP leads to the formation of hypoxanthine that diffuses out of the capillary endothelial cell (24). Hypoxia induced by exercise above maximal oxygen consumption also causes a mismatch between ATP supply and demand, resulting in the malfunction of

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ATP-dependent calcium pumps and intracellular accumulation of calcium (24). The increase in cellular calcium activates calcium-dependent proteases that lead to the proteolytic cleavage of a portion of xanthine dehydrogenase, converting it to xanthine oxidase (22). Recent work by Hellsten et al. (11) provides evidence for an increase in xanthine oxidase in human vascular cells of skeletal muscle after exercise. Xanthine oxidase then catalyzes the formation of xanthine from hypoxanthine and converts it to uric acid during intense exercise (12). These reactions use molecular oxygen as an electron acceptor and form the superoxide radical. The superoxide radical can combine with iron and form reactive hydroxy radicals that attack polyunsaturated fatty acids in cell membranes and initiate a chain of lipid peroxidation reactions that are the basis for part of the membrane disruption associated with exercise. Lipid peroxidation results in the formation of numerous aldehydes of different chain lengths, such as the 3-carbon product malondialdehyde (MDA), which has been shown to increase with dynamic resistance exercise (19, 20). The disruption to the cell membrane results in leakage of cytosolic proteins into the circulation, such as creatine kinase (19), myoglobin, and fatty acid-binding protein (FABP; see Ref. 26).

The primary purpose of this study was to examine the effects of L-carnitine L-tartrate (LCLT) supplementation on the magnitude of skeletal muscle disruption associated with the performance of moderate-intensity concentric/eccentric squat exercise. We measured several intermediate and end-point biochemical markers to assess the validity of our working hypothesis that carnitine, acting via an endothelial-mediated blood flow regulatory mechanism, could reduce catabolism of purines, free radical formation, sarcolemma disruption, and perceived soreness.

METHODS

Experimental design. This study involved a balanced, crossover, placebo-controlled research design that examined the effects of LCLT on markers of recovery after concentric/eccentric squat exercise. Subjects were matched for pretesting clinical values, activity background, nutritional patterns, and body size and then randomly assigned to either an LCLT or placebo group in a double-blind fashion. After 3 wk of supplementation (2 g L-carnitine/day), fasting morning blood samples were obtained on six consecutive days (D1-D6). Subjects performed a concentric/eccentric squat protocol (5 sets of 15-20 repetitions) on D2. During the squat protocol, blood samples were obtained preexercise and 0, 15, 30, 120, and 180 min postexercise. After a 1-wk washout period, subjects consumed the other supplement for a 3-wk period and performed the exact same procedures.

Subjects. Ten healthy, recreationally weight-trained men with a mean $\pm$ SD age 23.7 $\pm$ 2.3 yr, weight 78.7 $\pm$ 8.5 kg, and height 179.2 $\pm$ 4.6 cm served as subjects. All subjects were required to be engaged in a weight-training program that included the squat exercise for 1 yr before the study to exclude individuals that would experience a high degree of damage to the quadriceps in response to squatting for the first time. Subjects continued their normal training with the exception of the time period between D1 and D6 during which they were not allowed to train. All subjects were informed of the purpose and possible risks of this investigation before signing an informed consent document approved by an institutional review board.

Supplementation protocol. Subjects were provided with capsules of either L-CARNIPURE LCLT (Lonza, Fair Lawn, NJ) containing 736 mg LCLT (500 mg L-carnitine and 236 mg L-tartrate) or an identical-looking placebo (powdered cellulose) with written instructions to consume two capsules with breakfast and lunch for a total dose of 2 g L-carnitine/day. Supplementation commenced 3 wk before the squat protocol and continued through recovery. This dose of carnitine was chosen to maximize plasma carnitine concentrations without exceeding the renal threshold for carnitine (8, 23).

Exercise protocol. The squat exercise protocol was performed on a Plyometric Power System (PPS; Lismore) previously described in detail (29). Briefly, the PPS allows only vertical movement of the bar. Linear bearings attached to either end of the bar allow it to slide up and down two steel shafts with a minimum of friction. We determined each subject's one-repetition maximum (1-RM) in the squat exercise 1 wk before any supplementation using standard procedures in our laboratory (17). Pilot studies involving different exercise loads and magnetic resonance imaging (MRI) scans were performed to determine an exercise intensity that would elicit muscle tissue disruption but not severe damage to maximize the potential for LCLT to reduce hypoxia-mediated biochemical responses to exercise stress. The exercise protocol was performed in the afternoon 3 h after lunch (reproduced by each subject during both exercise days) and 3 h after the last dose of carnitine on D2. After a standardized warm-up (5 min of cycling), subjects performed five sets of 15-20 repetitions of squat with a load equal to 50% of their previously determined 1-RM squat. There was a 2-min recovery between each set. The load was decreased if <15 repetitions were performed.

Perceived muscle soreness. Pain was assessed using a 10-cm linear visual analog scale with labels that corresponded to "no pain" and "extreme pain" on either end. Subjects marked their level of subjective pain along the continuum, and the distance was reported as the raw score. The visual analog method has been established as a reliable method for assessing pain (21).

Muscle tissue disruption. Direct assessment of muscle tissue disruption and repair was evaluated using MRI cross sections and spin-spin relaxation time of the thigh muscles before the exercise test and 1 and 4 days postexercise using methods previously described in detail (6). The same investigator did all of the measurements with a reliability of $R = 0.99$. Scans were collected using a 0.3-Tesla open MRI magnet (AIRIS II; Hitachi Medical Systems America, Twinsburg, OH), and areas were measured with the National Institutes of Health (NIH) Macintosh computer program, NIH Image 1.55b 20, a MacIntosh Quadra 800 computer, and a scanner (Microtek Scanner III). NIH Image 1.55b 20 uses pixels of light to determine the area of skeletal muscle where damage occurs.

Blood collections. Blood samples were obtained on six consecutive days at the same time of the morning after a 12-h overnight fast and abstinence from alcohol and strenuous exercise. The last dose of carnitine was consumed during lunch the day before each morning blood draw (~18-20 h earlier). Subjects reported to the laboratory between 7:00 and 9:00 AM and rested quietly for 10 min in the supine position, and ~30 ml of blood were obtained from an antecubital vein with a 20-gauge needle and Vacutainers. On exercise days, a flexible catheter was inserted in a forearm vein, which was kept patent with a constant saline drip (60 ml/h). Before all
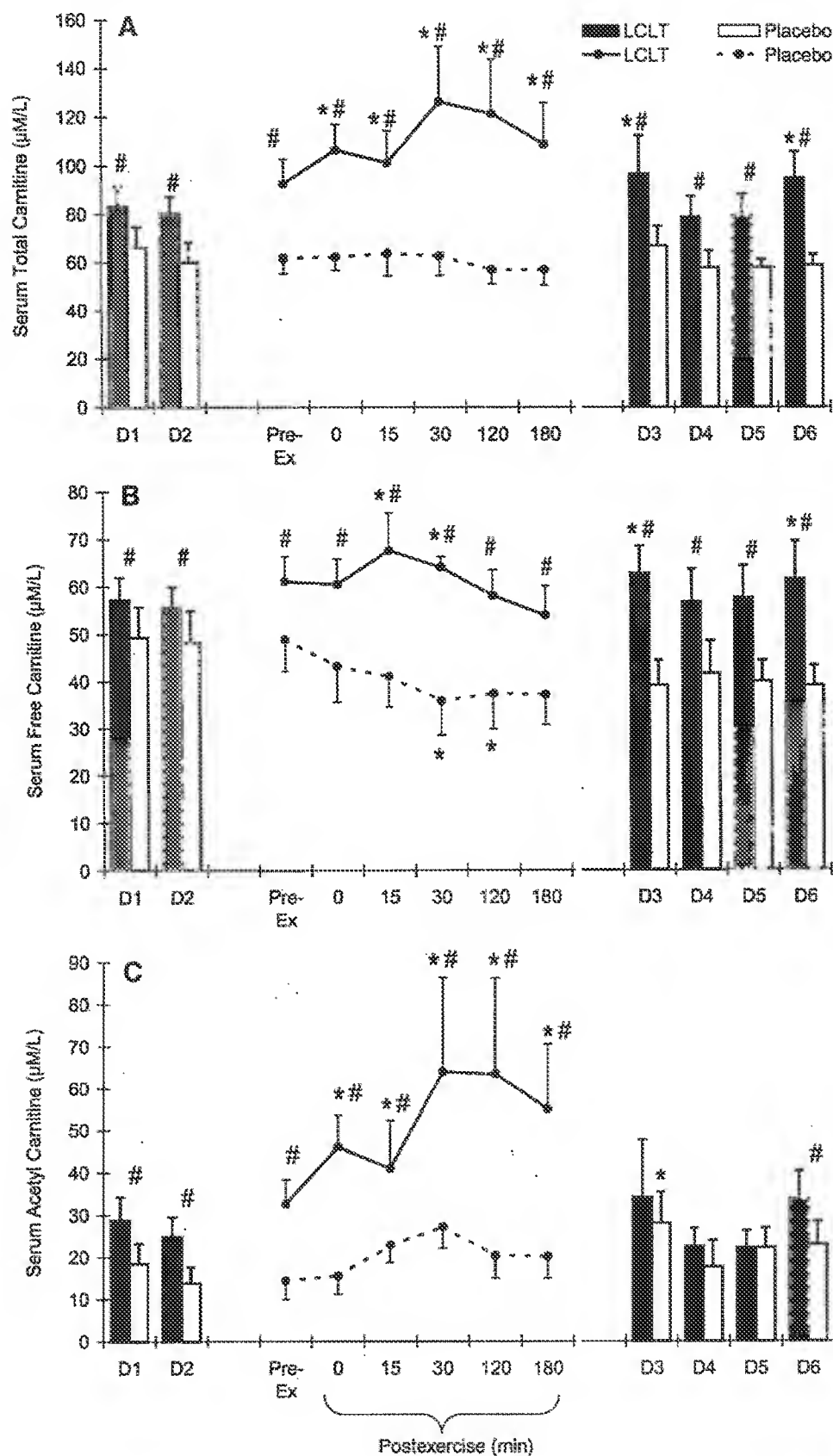
Fig. 1. Serum total carnitine (A), free carnitine (B), and acetylcarnitine (C) responses after 3 wk of either L-carnitine L-tartrate (LCLT) supplementation or placebo. Fasting morning blood samples were obtained in the morning for six consecutive days (D1-D6). Blood samples were also obtained preexercise and 0, 15, 30, 120, and 180 min after 5 sets of 15–20 repetitions of squat exercise on D2. *$P \le 0.05$ from corresponding preexercise value. #$P \le 0.05$ between corresponding LCLT and placebo values.
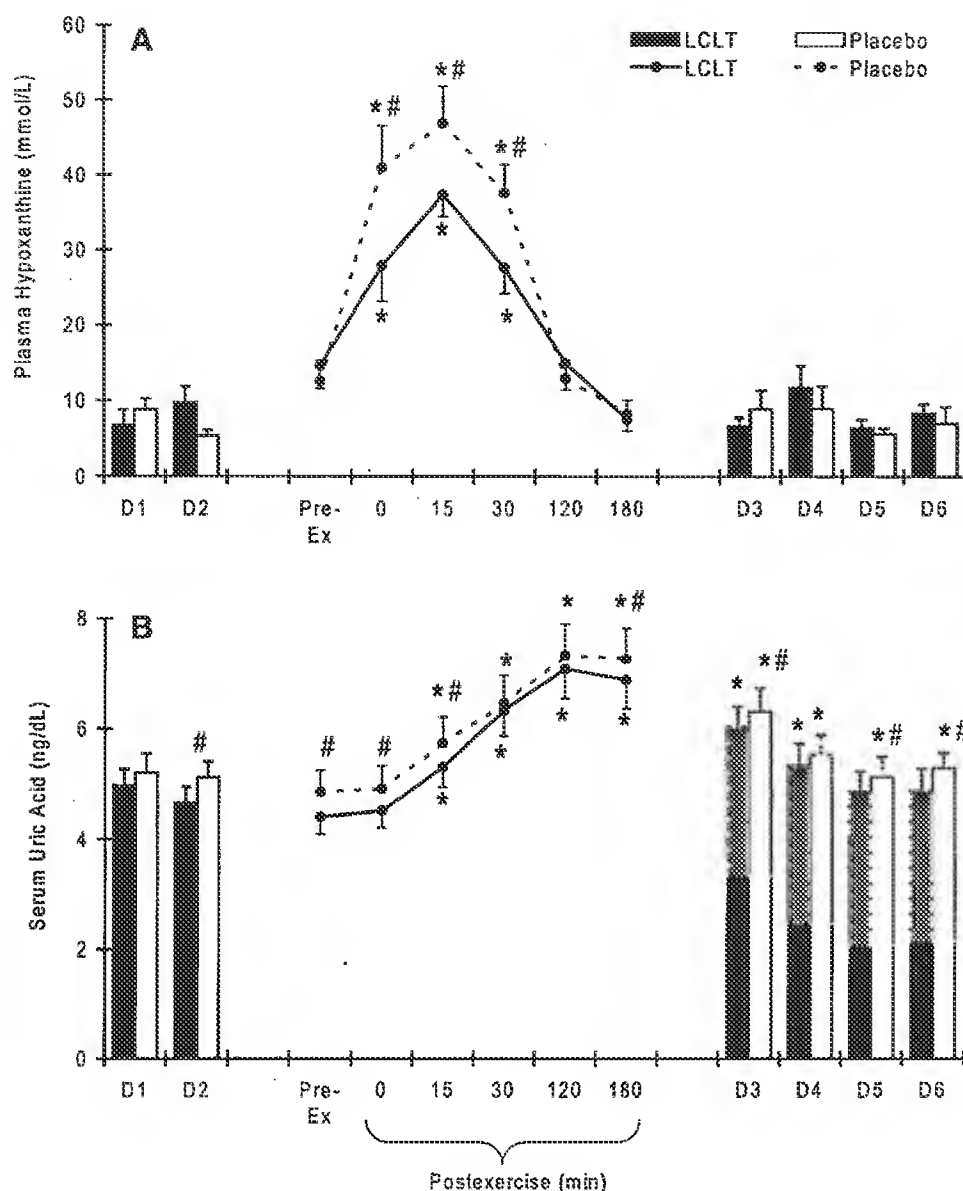
Fig. 2. Plasma hypoxanthine (A) and serum uric acid (B) responses after 3 wk of either LCLT supplementation or placebo. See Fig. 1 legend for description. $*P \leq 0.05$ from corresponding preexercise value. $\#P \leq 0.05$ between corresponding LCLT and placebo values.

blood collections, 3 ml of blood were withdrawn and discarded to avoid dilution of the sample, and ~30 ml were subsequently withdrawn and placed in two 10-ml tubes with a clot activator and one 10-ml tube containing EDTA. A 0.5-ml aliquot of whole blood was mixed with 1 ml of 0.8 M perchloric acid on ice for determination of plasma lactate. Within 15 min, whole blood was centrifuged (1,200 g for 15 min at 10°C), and the resultant serum/plasma was divided into aliquots and stored frozen at −80°C. Blood samples were collected preexercise and 0, 15, 30, 120, and 180 min postexercise. Subjects rested quietly in a seated position during the 3-h postexercise recovery period.

Blood analyses. Myoglobin was assayed in duplicate using a solid-phase ^{125}I-labeled RIA with a sensitivity of 10 ng/ml (American Laboratory Products, Windham, NH). Serum carnitine was assessed in the presence of acetyl-CoA by measuring the CoASH set free during acetyl transfer to carnitine by the enzyme carnitine acetyltransferase. The CoASH was trapped with 5,5′-dithiobis-(2-nitrobenzoic acid) and measured spectrophotometrically at 412 nm (28). Acetylcarnitine

was calculated by taking the difference between total carnitine and free carnitine. Plasma lactate and serum creatine kinase (Sigma Diagnostics, St. Louis, MO) were determined at 340 nm on a spectrophotometer (Spectronic 601; Milton Roy, Rochester, NY). Serum hypoxanthine and xanthine oxidase were analyzed in duplicate using the Amplex Red reagent-based assay (A-22182; Molecular Probes, Eugene, OR). After a 30-min incubation at 37°C, absorbances were read on a microplate reader (Wallac Victor; EG&G, Gaithersburg, MD) at 560 nm. The absorbance of the blank (zero standard) was subtracted from each data point to standardize for background absorbance. FABP was determined in plasma using a solid-phase enzyme-linked immunoabsorbent assay of the sandwich type (Hbt HK402; Cell Sciences, Norwood, MA). Plasma MDA was determined using the methods described by Wong et al. (30) and modified as described by McBride et al. (19). A phosphoric acid solution (0.44 mol/l) and a thiobarbituric acid solution (42 mol/l) were added to plasma samples and placed in a water bath (0°C) until analysis. A methanol-NaOH solution was added to the boiled
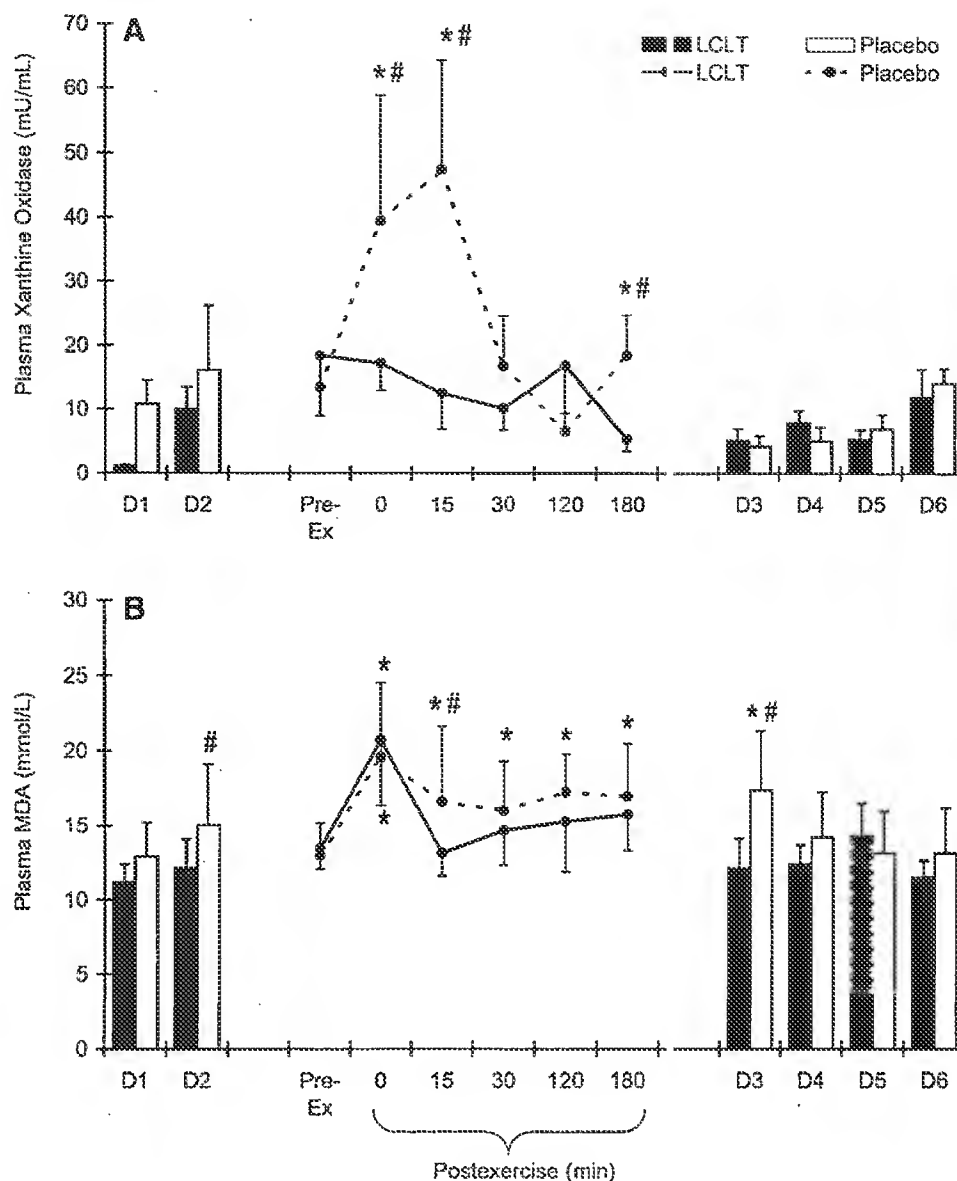
Fig. 3. Plasma xanthine oxidase (*A*) and plasma malondialdehyde (MDA; *B*) responses after 3 wk of either LCLT supplementation or placebo. See Fig. 1 legend for description. *$P \leq 0.05$ from corresponding preexercise value. #$P \leq 0.05$ between corresponding LCLT and placebo values.

samples before being centrifuged to precipitate the plasma proteins. The protein-free plasma was extracted, and the absorbance was read at 532 nm on a spectrophotometer. All samples were thawed only one time, and all intra-assay and interassay coefficients of variance for each analyte were <10%.

Statistical analyses. A two-way repeated-measures ANOVA was used to evaluate changes over time in the LCLT and placebo conditions. When a significant *F*-value was achieved, the Tukey's post hoc tests were used to locate the pairwise differences between means. The total area (serum concentration × time) under the line connecting pre- and postexercise biochemical concentrations was calculated using the trapezoidal method. The level of significance was set at $P \leq 0.05$.

RESULTS

Serum carnitine. Compared with placebo, serum total, free, and acetylcarnitine concentrations were significantly higher during LCLT at all time points measured with the exception of acetylcarnitine values

during D3, D4, and D5. Free carnitine was significantly reduced at 30 and 120 min postexercise during placebo, whereas free carnitine was significantly increased at 15 and 30 min postexercise during LCLT. The acetylcarnitine response to exercise was unchanged during placebo, whereas acetylcarnitine was significantly increased from 0 to 180 min postexercise during LCLT (Fig. 1).

Lactate response. There were significant increases in plasma lactate that peaked immediately after exercise (>10 mmol/l) and returned to preexercise concentrations by 120 min postexercise. The lactate responses were not significantly different between LCLT and placebo conditions.

Purine catabolism. Plasma hypoxanthine concentrations were significantly elevated above preexercise values up to 30 min postexercise during LCLT and placebo. The exercise-induced responses were significantly greater during placebo. Preexercise serum uric
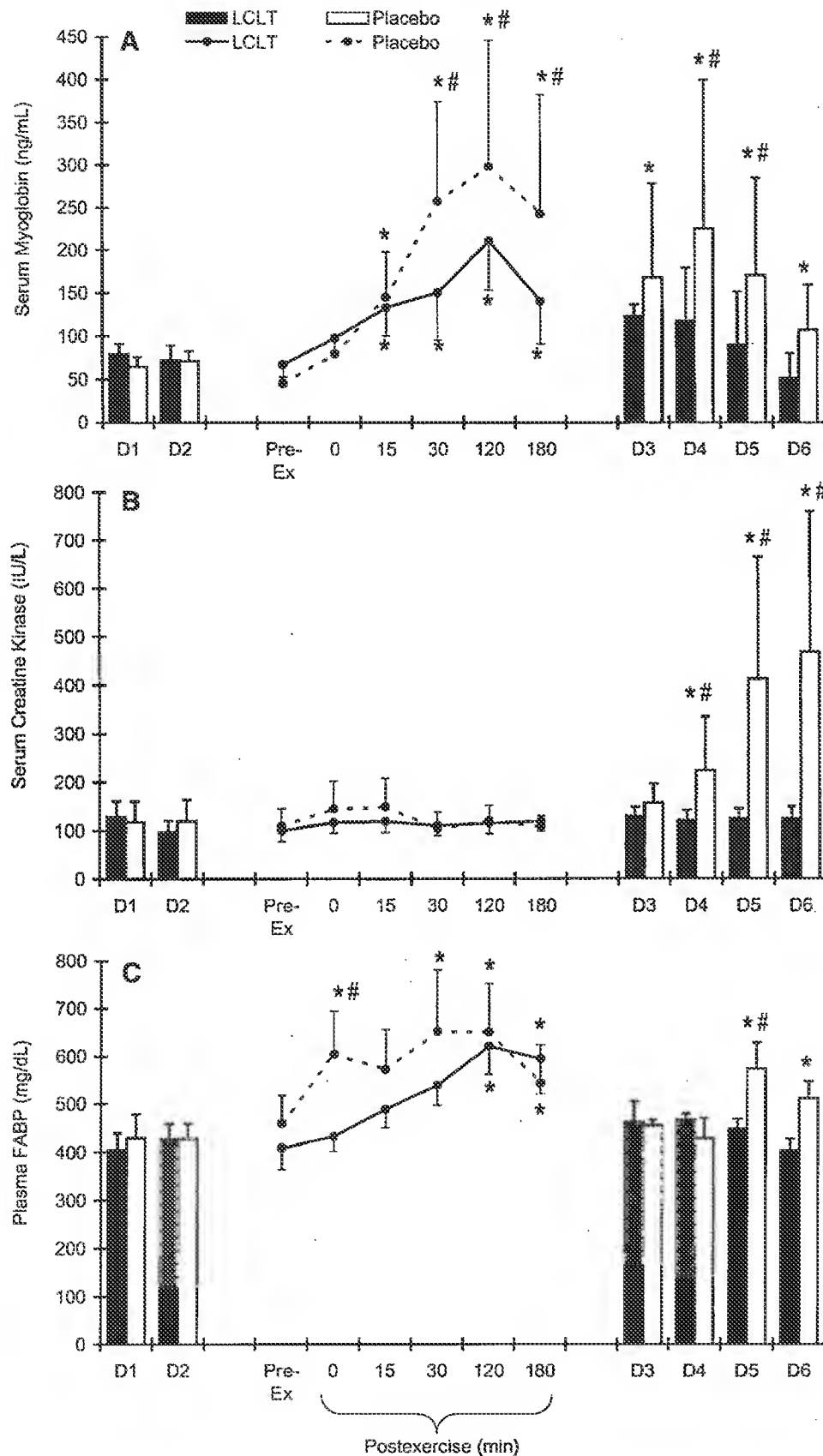
Fig. 4. Serum myoglobin (A), serum creatine kinase (B), and plasma fatty acid-binding protein (FABP; C) responses after 3 wk of either LCLT supplementation or placebo. See Fig. 1 legend for description. *$P \leq 0.05$ from corresponding preexercise value. #$P \leq 0.05$ between corresponding LCLT and placebo values.

acid concentrations were significantly higher during the placebo vs. the LCLT condition. There were significant increases in uric concentrations that peaked ~120–180 min after exercise. Uric acid concentrations remained higher during placebo throughout the 4-day recovery period (D3–D6; Fig. 2).

Free radical generation. Plasma xanthine oxidase concentrations were not significantly different at any time point during LCLT but significantly increased above preexercise values at 0, 15, and 180 min postexercise during placebo. Plasma MDA responses peaked immediately after exercise during both LCLT and placebo. Plasma MDA returned to resting values by 15 min postexercise during LCLT, whereas MDA remained significantly elevated above preexercise throughout 24 h of recovery during placebo (Fig. 3).

Cytosolic proteins. Serum myoglobin concentrations peaked at 120 min postexercise, and the response was significantly greater during placebo. Serum myoglobin returned to preexercise concentrations the day after exercise during LCLT but remained significantly elevated throughout the 4-day recovery period during placebo. Serum creatine kinase activity was not significantly increased at any time point during LCLT. However, creatine kinase began to accumulate above baseline levels 2 days after exercise (D4) and continued to increase further 3 (D5) and 4 (D6) days after exercise during placebo. Plasma FABP responses to exercise were variable, increasing immediately after exercise during placebo and at 120 min postexercise during LCLT. Compared with LCLT, corresponding FABP values in the placebo group were significantly higher immediately after exercise and on the third day of recovery (D5; Fig. 4).

Muscle disruption and perceived soreness. The percentage of muscle tissue disruption assessed from MRI cross-sectional scans of the midthigh at D3 and D6 were 23 ± 8 and 16 ± 5% for LCLT and 39 ± 5 and 29 ± 6% for placebo, respectively. The percent muscle tissue disruption was significantly greater during placebo. Perceived muscle soreness peaked the day after exercise and continued to decline throughout the 4 days of recovery. Compared with corresponding values for LCLT, soreness ratings were significantly higher 1, 2, and 4 days after exercise during placebo (Table 1).

DISCUSSION

The mechanical and biochemical stress responses to exercise are complex and involve activation of the adenylate kinase system, generation of reactive oxygen species, inflammatory responses, and disruption to the contractile apparatus and cell membranes that ultimately contribute to fatigue by adversely impacting the metabolic, structural, and functional integrity of muscle. Similar to ischemia-reperfusion, intense exercise creates a scenario for certain active muscle fibers that can lead to transient or prolonged episodes of hypoxia followed by reoxygenation at the cessation of exercise. In addition, high rates of ATP turnover contribute to a mismatch between ATP supply and de-

Table 1. *Ratings of perceived muscle soreness*

	LCLT	Placebo
Preexercise (baseline)	0.0 ± 1.1	0.1 ± 0.2
Postexercise		
Day 1	4.3 ± 1.6*	5.8 ± 1.8*†
Day 2	4.9 ± 1.8*	5.5 ± 1.7*†
Day 3	3.? ± 1.2*	2.1 ± 1.3*
Day 4	1.0 ± 1.2	1.8 ± 0.6*†

Values are means ± SD. Pain was assessed using a 10-cm linear visual analog scale (1 = no pain; 10 = extreme pain). LCLT, L-carnitine L-tartrate. *$P \leq 0.05$ from corresponding preexercise value. †$P \leq 0.05$ from corresponding LCLT value.

mand. To examine the potential for LCLT supplementation to impact recovery from exercise, we measured indicators of adenine nucleotide degradation (hypoxanthine and uric acid), free radical formation (MDA and xanthine oxidase), cell membrane damage (creatine kinase, FABP, and myoglobin), muscle disruption/damage, and perceived muscle soreness. A major assumption of our working hypothesis was that LCLT supplementation would lead to an accumulation of carnitine in endothelial cells (or prevent carnitine deficiency) and thereby increase blood flow, oxygen delivery, and regeneration of ATP. In general, the results support our hypothesis that LCLT would attenuate the biochemical and structural stress responses to high-repetition squat exercise.

Serum total carnitine concentrations were significantly higher during all time points of the study during LCLT supplementation, indicating good compliance with the supplementation protocol. During placebo, there was no change in the total carnitine response to exercise; however, free carnitine was significantly reduced at 30 and 120 min postexercise, and there was a slight increase in acetylcarnitine. A decrease in serum free carnitine and an increase in acetylcarnitine after intense exercise is in agreement with other studies that have examined carnitine responses to exercise (1, 9). Although there are proportional changes in free and acetylcarnitine in muscle during exercise, it is generally accepted that the total muscle carnitine concentration is not altered during exercise and does not interact significantly with carnitine metabolism in the circulation (10).

Plasma lactate responses to squat exercise were significantly increased (>10 mmol/l) to a similar extent during LCLT and placebo, indicating a similar glycolytic and acid-base stress between conditions. High glycolytic rates are associated with accumulation of ADP and H^+, which favor activation of adenylate kinase and the formation of ATP and AMP from two molecules of ADP. In muscle, AMP is oxidized to hypoxanthine. Indeed, plasma hypoxanthine was significantly elevated above preexercise values during the early postexercise period, and the response was attenuated by LCLT supplementation, indicating a better match between ATP supply and demand.

During strenuous exercise, the inadequate generation of ATP can cause malfunctioning of calcium

ATPase pumps and an increase in intracellular calcium, which in turn activates calcium-dependent proteases. These proteases cleave a portion of xanthine dehydrogenase, converting it into xanthine oxidase, which utilizes molecular oxygen as an electron acceptor instead of NADH. Xanthine oxidase results in the generation of superoxide radicals during exercise (13), which can initiate lipid peroxidation and cell disruption/damage (4). In this study, plasma xanthine oxidase was significantly elevated above preexercise values during the 0 and 15 min postexercise time points during the placebo condition, whereas the exercise-induced response was eliminated during LCLT. The conversion of xanthine dehydrogenase to xanthine oxidase occurs via calcium-dependent proteases in the cytosol (22). The significantly lower xanthine oxidase after exercise during LCLT indirectly suggests that either calcium pumps were operating more efficiently (perhaps because of better ATP supply) and/or there was less disruption to the muscle/sarcoplasmic reticulum membrane that would cause ionic disturbances resulting in accumulation of calcium in the cytosol.

Inhibition of xanthine oxidase with allopurinol during exercise has been shown to result in significantly less generation of reactive oxygen species, less accumulation of cytosolic enzymes (creatine kinase and lactate dehydrogenase), and less tissue damage after exhaustive exercise (4, 13, 27). In this study, the lower xanthine oxidase response during LCLT was also associated with less accumulation of reactive oxygen species (as measured by MDA), less accumulation of cytosolic proteins (as measured by circulating creatine kinase activity, myoglobin, and FABP), less tissue damage (as measured by MRI), and less subjective muscle soreness after squat exercise.

Generation of the superoxide radical during intense exercise (presumably via endothelial xanthine oxidase) could initiate a series of tissue-destructive reactions, including lipid peroxidation. We used MDA as a quantitative marker for free radical interaction with cell membranes. There was a significant increase in plasma MDA to the squat exercise protocol that peaked immediately after exercise. We previously demonstrated that a whole body resistance exercise protocol resulted in significant increases in plasma MDA immediately postexercise (19). Plasma MDA returned to preexercise values by 15 min postexercise during LCLT but remained significantly above preexercise values for >180 min postexercise during placebo. The overall lower MDA response to the squat protocol indicates that LCLT resulted in less total exposure of cell membranes to the damaging effects of reactive oxygen species. Increased generation of reactive oxygen species would be predicted to cause greater disruption/damage to the sarcolemma and greater leakage of cytosolic proteins into the circulation. The extent of muscle damage measured using MRI and the accumulation of cytosolic proteins in the circulation were significantly reduced by LCLT, providing further evidence for a protective effect of LCLT on muscle disruption/damage.

It has been proposed that FABP and myoglobin represent more useful markers for the early detection of exercise-induced muscle damage because of their relatively rapid response, whereas the creatine kinase response to exercise occurs at a slower rate (26). In the acute 3-h recovery period after exercise, we observed significantly lower postexercise myoglobin and FABP responses during LCLT. Creatine kinase activity was not increased during LCLT or placebo during the 3-h recovery period but progressively increased above baseline beginning 2 days after exercise during placebo. The lack of a creatine kinase response to exercise during LCLT is indicative of less disruption to the sarcolemma. Similar to our findings, Giamberardino et al. (7) demonstrated that the creatine kinase responses to a 20-min eccentric step test were attenuated in subjects that consumed 3 g carnitine/day for 3 wk before exercise.

Our data provide indirect evidence of a favorable effect of LCLT supplementation (2 g L-carnitine/day for 3 wk) on endothelial blood flow regulation during and after moderate-intensity squat exercise, as evidenced by significantly less accumulation of markers of purine degradation, free radical formation, tissue damage, and muscle soreness. Neither endothelial carnitine concentrations nor local blood flow was measured in this study to add more direct support to our hypothesis. However, the data do favor a significant effect of LCLT on recovery stress responses acting via a cell/tissue-related mechanism. We favor the vascular compartment as the target for the beneficial effect of carnitine on exercise recovery responses.

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EXHIBIT 4

Characterization of Acylcarnitines as Their Isobutyl Ester Derivatives Using Fast Atom Bombardment Mass Spectrometry and Constant Neutral Loss Scan

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A simple method is described for the characterization of acylcarnitines by fast atom bombardment mass spectrometry in combination with constant neutral loss (CNL) scan. Acylcarnitines in urine are extracted using cation-exchange chromatography and derivatized to their isobutyl esters. The fragmentation patterns of these compounds are studied by various linked-scan methods including B/E, B^2/E and $B/E\sqrt{(1-E)}$ scans. Acylcarnitine isobutyl esters are found to lose two specific neutral fragments simultaneously so that a CNL scan of the combined mass produces a much enhanced spectrum without most of the background peaks. An example is given in which the CNL scan is used to diagnose a patient with medium-chain acyl CoA dehydrogenase deficiency. The use of linked-scan techniques to monitor a specific metastable ion transition can be exploited for characterizing structurally related compounds and therefore the technique reported in this paper may have much wider applications to mixture analysis.

INTRODUCTION

Recent studies have shown that abnormalities in urinary excretion of acylcarnitines are associated with many disorders of organic acid metabolism in which acyl CoA esters accumulate in the mitochondria.[1-3] These acylcarnitines are formed in the mitochondria by the transesterification of the accumulating acyl CoA esters with L-carnitine, via the action of carnitine acyl-transferases, in order to remove these potentially toxic acyl moieties and to restore mitochondrial homoeostasis.[1] The identity of these acylcarnitines is thus of considerable biochemical and diagnostic importance.

Although these quaternary amino compounds are easily ionized by fast atom bombardment (FAB) giving prominent molecular ions, trace analysis of these metabolites in urine, normally excreted at micromolar levels, requires additional characterization such as linked B/E scan and accurate mass measurements.[2] Recently tandem mass spectrometry has also been applied to the differentiation of certain isomeric acylcarnitines.[4]

On a double-sector instrument, metastable ion transitions occurring in the field-free region (FFR) can be monitored by scanning the electrostatic and magnetic sectors simultaneously.[5] This provides additional information on ion fragmentation and is particularly useful for 'soft' ionization techniques such as FAB. Within the limits of instrumental resolution, these often characteristic metastable ion transitions can be exploited to improve the specificity of the analysis.

In this paper we report the use of FAB mass spectrometry in combination with the constant neutral loss (CNL) scan to identify acylcarnitine mixtures as their isobutyl ester derivatives. The major metastable ion transitions of acylcarnitine (C_2-C_{10}) isobutyl esters were also studied by B/E, B^2/E and $B/E\sqrt{(1-E)}$ linked-scan modes. The fragmentation patterns obtained were compared with those of the underivatized acylcarnitines. The potential use of these linked-scan methods in the identification of acylcarnitines is discussed. It has also been demonstrated in this study that constant neutral loss scan is a useful technique for removing background peaks in the FAB spectrum, which are often a problem in trace analysis

EXPERIMENTAL

Carnitine and acylcarnitines (C_2-C_{10}) were obtained from either Sigma Chemical Co. Ltd (Poole, UK) or P-L Biochemicals Inc. (Milwaukee, Wisconsin, USA). Acylcarnitines in urine (typically 1 ml) were extracted using Dowex 50 cation-exchange resin followed by freeze-drying the eluate as described previously.[6]

The isobutyl ester derivatives of acylcarnitines were prepared using a modified method by Brenner and Huber.[7] The sample was dissolved in isobutanol (1 ml), cooled to $-70\,^\circ$C in a solid CO_2–acetone bath and then mixed with thionyl chloride (400 µl). The mixture was allowed to warm slowly to 45 °C and maintained at this temperature for 30 min. Excess reagents were then removed under nitrogen. The residue was redissolved in 20–50 µl of methanol ready for mass spectrometric measurement.
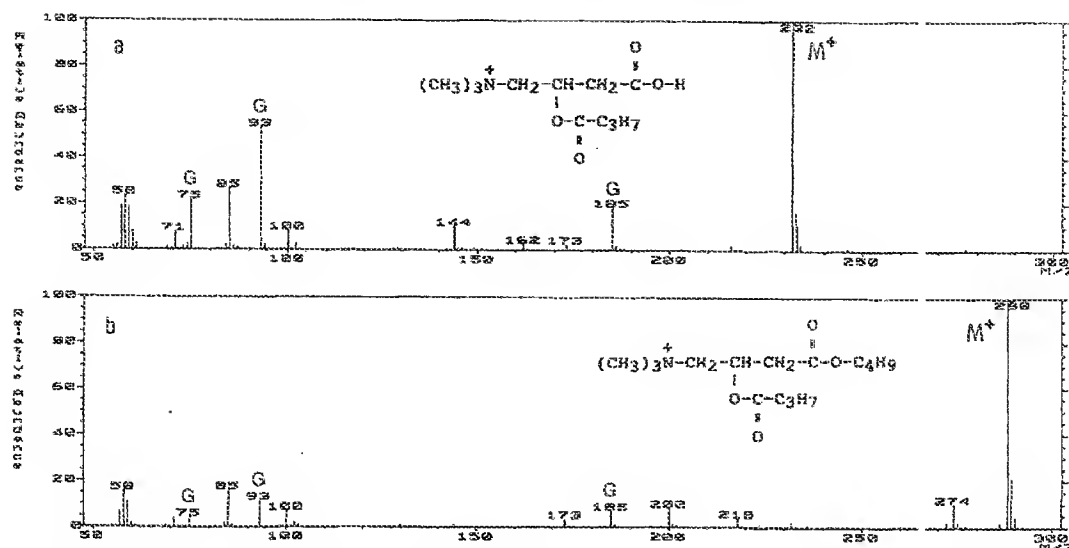
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Figure 1. FAB spectra of (a) n-butyrylcarnitine and (b) its isobutyl ester. Peaks labelled 'G' are glycerol matrix ions.

The mass spectrometer used was a JEOL DX-303 forward-geometry double-sector instrument equipped with a JEOL DA-5000 data system. Acylcarnitines and their isobutyl esters were ionized by FAB using a xenon beam of 6 keV energy. The sample containing approximately 1 µg of acylcarnitines was applied onto the FAB target and glycerol was used as the sample matrix.

Metastable ion transitions in the 1st FFR were recorded by various linked-scan methods, including B/E

(daughter ion scan), B^2/E (parent ion scan) and $B/E\sqrt{(1 - E)}$ (CNL scan). Collision-induced dissociation (CID) was employed in the B/E scan mode by introducing helium into the collision chamber in the 1st FFR at a pressure to give a 50% decrease in the precursor ion abundance. The resolution of the mass spectrometer was set to 500 for all measurements, but when accurate mass determinations were used to confirm the identity of certain fragment ions the resolution was set to 3000
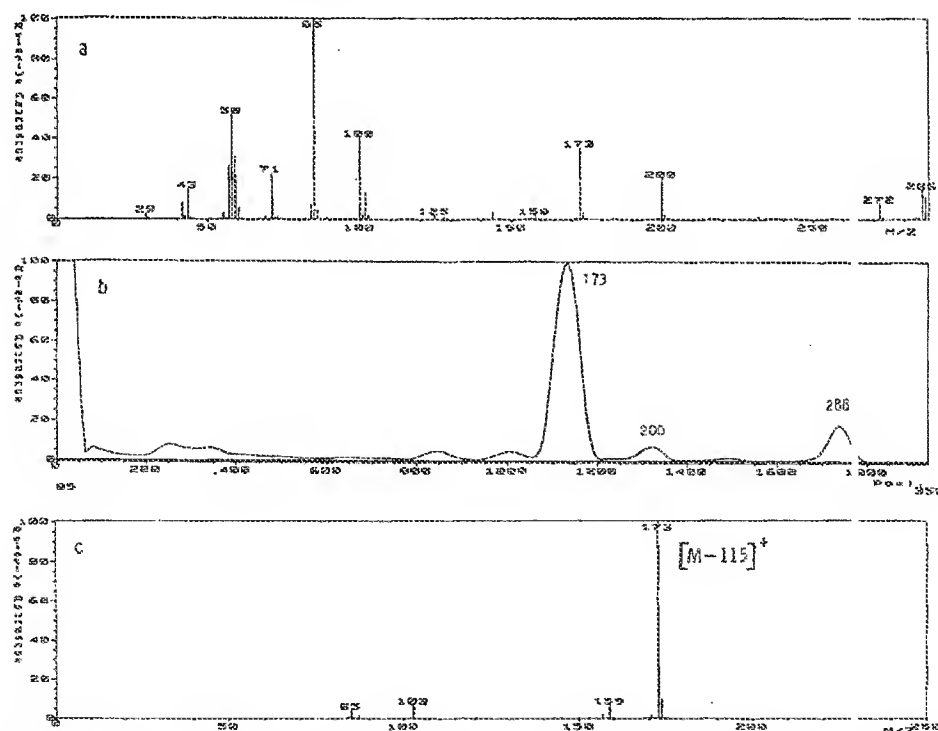

Figure 2. Butyrylcarnitine isobutyl ester spectra: (a) daughter ion scan of m/z 288; (b) precursor ion scan of m/z 85; and (c) CNL of [m - 115]+.

and the calculations were made using glycerol–PEG 200 (1:1 v/v) matrix ions or other ions of known composition as reference.

RESULTS AND DISCUSSION

Carnitine and its acyl conjugates were found to give very intense positive ion FAB spectra owing to the presence of quaternary nitrogen.[1,2] Upon esterification of the carboxyl group a significant improvement in ion yield was observed, probably reflecting the increase in ionization efficiency due to the loss of zwitterionic structure.

The FAB spectra in all cases showed abundant molecular ions as exemplified by the spectra of n-butyryl carnitine and its isobutyl ester derivative, shown in Fig. 1. B/E scan of the molecular ion of the isobutyl ester (m/z 288) under CID conditions gave daughter ions (Fig. 2(a)) similar to those from the underivatized n-butyrylcarnitine which have been published previously.[2] Daughter ions which were common to both the free acid and the isobutyl ester were found at m/z 58, 85, 100 and $[M - 88]^+$. The structure of these fragment ions can be found in Fig. 3.

Unlike the free acid and methyl ester,[4] the isobutyl ester did not show a $[M - 59]^+$ fragment ion corresponding to the loss of $(CH_3)_3N$ group or the daughter ion corresponding to the fragment of $^+CH_2CH=CHCOOC_4H_9$ (see Figs 2(a) and 3).

Precursor ion scan (B^2/E) of the principal daughter ion at m/z 85 indicated that it was derived predominantly from m/z 173 (Fig. 2(b)). This in turn was found

Table 1. Accurate mass measurements of some major fragment ions from n-butyrylcarnitine isobutyl ester

Observed mass	Fragment ion	Error (ppm)
58.0656	C_3H_8N	-2
85.0300	$C_4H_5O_2$	+3
100.1130	$C_6H_{14}N$	+4
173.0810	$C_8H_{13}O_4$	-2
200.1640	$C_{11}H_{22}NO_2$	-5

to be derived directly from the molecular ion (m/z 288) in another B^2/E scan. This was also confirmed by the CNL scan of the neutral fragment (115 daltons) as shown in Fig. 2(c).

This characteristic loss of 115 daltons from the molecular ion might either correspond to the single loss of $-CH_2COOC_4H_9$ fragment or the concerted loss of $(CH_3)_3N$ and C_4H_8 from the molecular ion. In order to distinguish these two possible mechanisms accurate mass determination of all major daughter ions was carried out, and the results are shown in Table 1.

From the accurate mass measurements, it was confirmed that the neutral loss of 115 daltons from the molecular ion corresponded to the concerted loss of $(CH_3)_3N$ and C_4H_8 fragments and not the single-step mechanism as we originally proposed.[8] This was interesting because neither $[M - (CH_3)_3N]^+$ nor $[M - C_4H_8]^+$ ion was observed in the daughter ion spectrum.

Detailed study of the metastable ion transitions using C_2–C_{10} acylcarnitine isobutyl esters (see Table 2) indicated that they all showed an identical fragmentation
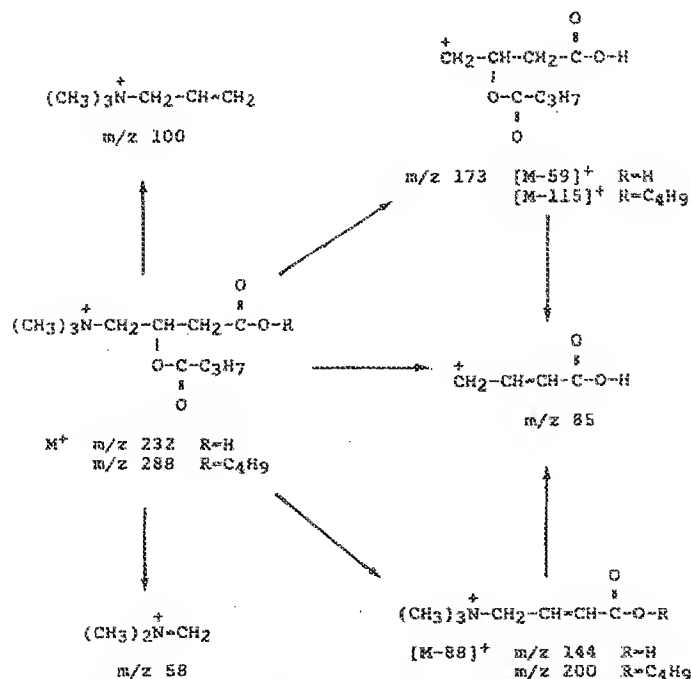

Figure 3. Major fragmentation pathways for free (R=H) and esterified (R=C$_4$H$_9$) acylcarnitines as exemplified by n-butyrylcarnitine.

Table 2. The major daughter ions of acylcarnitine isobutyl esters obtained by CID of the molecular ions

Acyl chain	M⁺·	Daughter ion, m/z (%)*
Acetyl	260	58(57), 85(100), 100(48), 145(41), 200(16)
Butyryl	288	58(52), 85(100), 100(41), 173(36), 200(21), 272(8)
Hexanoyl	316	58(33), 85(100), 100(26), 200(23), 201(56), 272(20)
Octanoyl	344	58(40), 85(100), 100(28), 200(33), 229(34), 272(39)
Decanoyl	372	58(12), 85(71), 100(15), 200(27), 257(100), 272(41)

* These figures are expressed relative to the principal daughter ion (100%).

pattern as shown in Fig. 3. The neutral loss of 115 daltons is very specific to acylcarnitine isobutyl esters, giving a much simplified neutral loss spectrum with the daughter ion $[M - 115]^+$ still retaining the acyl chain. The CNL scan was applied to a urine sample spiked with acylcarnitines (C_3–C_{10}) at approximately 10 µg ml⁻¹ (100 ng of sample applied on-target). Figure 4(a) and (b) compares the normal FAB spectrum of this sample with that obtained from the CNL scan, showing the improvement in the specificity and the quality of the spectrum.

Figure 4(c) shows another example of a CNL scan of a urine sample obtained from a patient who was diagnosed as having medium-chain acyl CoA dehydrogenase deficiency characterized by the large amount of octanoylcarnitine excreted in the urine.[1] The urine sample was also analysed for organic acids by gas chromatography/mass spectrometry (GC/MS), showing the presence of suberylglycine and medium-chain dicarboxylic acids which confirmed this diagnosis.

These results exemplify the way in which the linked-scan techniques are useful for the identification of a series of structurally related compounds in a crude mixture by monitoring a specific metastable ion transition. The CNL scans shown here not only serve to characterize acylcarnitine peaks present in the FAB spectrum but also help to remove those 'background' peaks, resulting in a much enhanced spectrum. One limitation of this method is that it cannot be used to distinguish structural isomers which can exist on the acyl chain.

In principle the same method can be applied to the underivatized acylcarnitines by scanning the neutral loss of 59 daltons, although this neutral fragment is neither specific nor sensitive enough to give any significant improvement over the normal FAB spectrum.

We have discussed so far only the qualitative application, although quantification of acylcarnitines by this method is possible using isotope dilution analysis with labelled acylcarnitines as internal standards. Initial
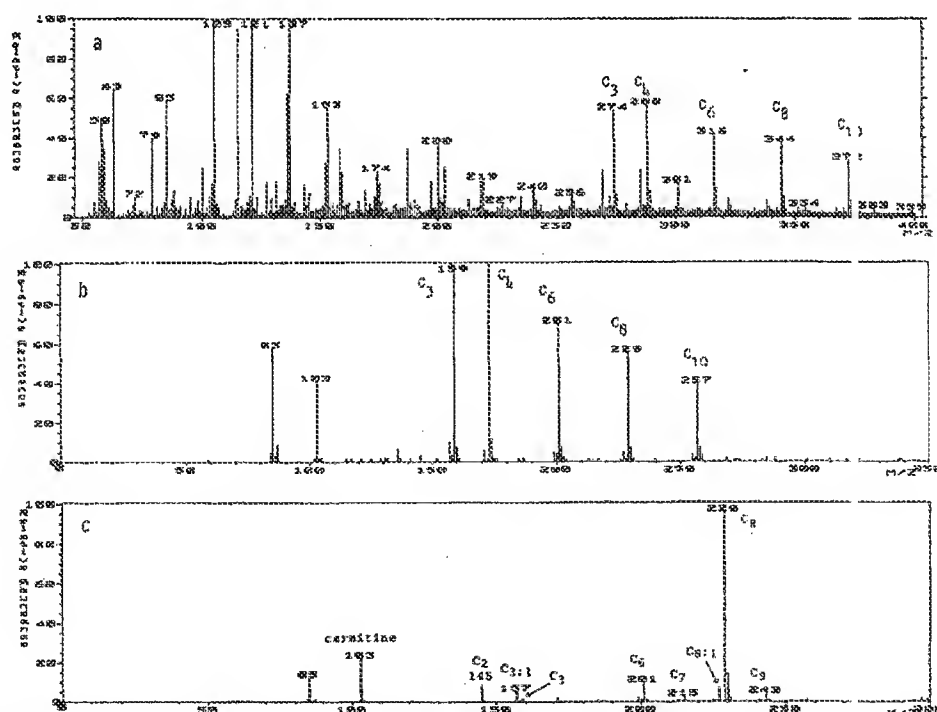

Figure 4. Acylcarnitines extracted from 'spiked' urine sample and esterified: (a) normal FAB spectrum and (b) constant neutral loss of $[m - 115]^+$. (c) The CNL scan of the urine sample from a patient with medium-chain acyl CoA dehydrogenase deficiency, showing the intense octanoylcarnitine isobutyl ester.
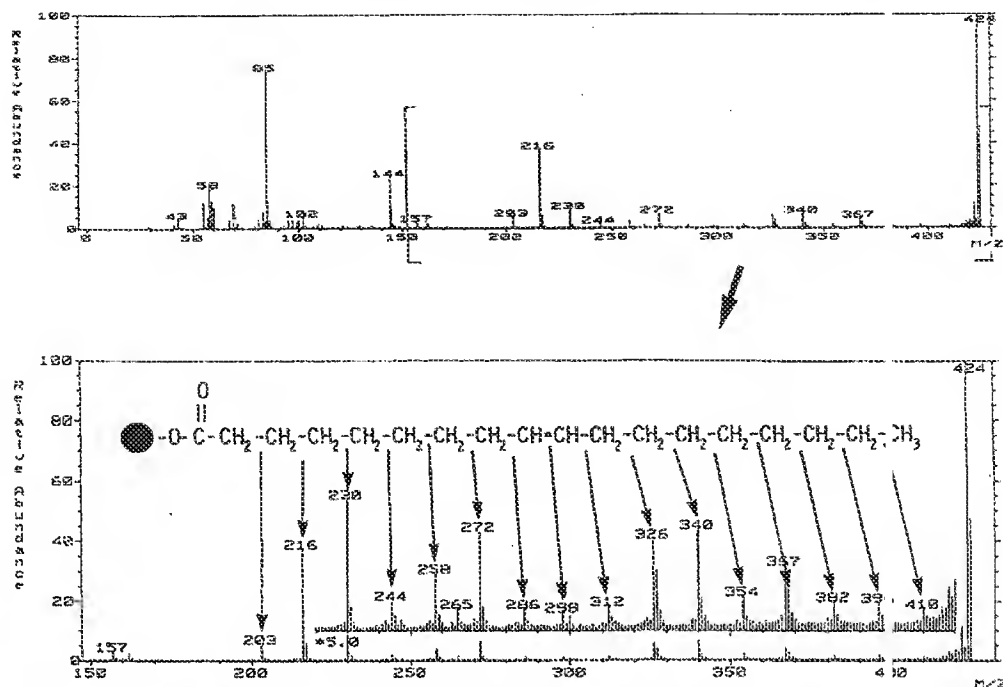
Figure 5. CID *B/E* scan of oleoylcarnitine, showing C—C bond cleavage of the acyl chain and the position of double bond. '●' represents the carnitine moiety.

results using acetyl- and propionylcarnitine and their deuterated analogues as internal standards gave linear calibration curves over more than three orders of magnitude.[9] The same neutral loss scan is applicable to the analytes and their internal standards provided that the label site is not on the neutral fragments.

One major advantage of the CNL scan over the *B/E* scan is that all acylcarnitines in the sample can be characterized in a single run. Although the signal intensities obtained in linked scans are usually one to two orders of magnitude lower than those from the normal scans, the increase in specificity often results in better signal to noise ratio.

The presence of 'artefact' peaks in linked scanning of a double-sector instrument[10] can sometimes cause ambiguity in the interpretation of data, although this did not appear to be a problem in this study. Another potential problem with linked scanning on a double-sector instrument is that both *B/E* and *B²/E* scans suffer from poor precursor ion resolution and that CNL scan

suffers from poor fragment loss resolution. These limitations, however, are not associated with a multi-stage quadrupole system, where the analysis of these acylcarnitines may be performed by monitoring all the precursor or molecular ions which produce a specific daughter ion in order to obtain an 'acylcarnitine profile'-like spectrum.

Finally, it was also found that the CID *B/E* spectra of acylcarnitines with an acyl chain length longer than C_4 gave a series of ions based on m/z 216 (m/z 272 for the isobutyl esters) in increasing increments of 14 daltons (Fig. 5). These ions presumably correspond to $[●-OCO(CH_2)_nCH=CH_2]^+$, where ● is the carnitine moiety, and represent the C—C bond cleavage along the acyl chain, which will be useful in locating the position of a double bond in unsaturated acylcarnitines.

Acknowledgement

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